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ORIGINAL ARTICLE

A *Dehalococcoides*-containing co-culture that dechlorinates tetrachloroethene to *trans*-1,2-dichloroethene

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In the microbial reductive dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE), dechlorinators usually produce *cis*-1,2-dichloroethene (*cis*-DCE) as the predominant product or an intermediate. This study shows that dechlorination of PCE and TCE can also lead to the generation of *trans*-1,2-dichloroethene (*trans*-DCE) by a co-culture MB. During its enrichment process, the ratio of *trans*- to *cis*-DCE increased from 1.4 (±0.1):1–3.7 (±0.4):1, whereas the TCE reductive dechlorination rate went up from ~26.2 to ~68.8 μmol l^{-1} day^{-1}. PCR–denaturing gradient gel electrophoresis (PCR-DGGE) revealed that the increased ratio of *trans*- /*cis*-DCE was well correlated with the increased proportions of *Dehalococcoides* and the disappearance of *Desulfuromonas* during the enrichment process. As shown by PCR-DGGE, similar *Dehalococcoides* species were consistently present in another three sediment-free cultures with various *trans*- /cis-DCE ratios. The 16S rRNA gene sequence of this *Dehalococcoides* sp. in co-culture MB is 100% identical (over 1489 bp) to that of *Dehalococcoides ethenogenes* strain 195 (CP000027), which belongs to the Cornell subgroup of the *Dehalococcoides* cluster. The other bacterium in this co-culture MB was a *Sedimentibacter* species, which showed no PCE or TCE dechlorination activity. Results from this study show that microbial dechlorination of chloroethenes by this particular subgroup of *Dehalococcoides* could result in significant accumulation of *trans*-DCE in the environment if no *trans*-DCE dechlorinators coexist in the contaminated sites.
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Introduction

Large quantities of chlorinated organic solvents tetrachloroethene (PCE) and trichloroethene (TCE) have been released into the environment because of their widespread usage (for example, metal degreasing or dry cleaning) (Abelson, 1990). Under anaerobic conditions, PCE and TCE can be dechlorinated to dichloroethenes (DCEs) by a variety of microbes (for example, *Dehalobacter, Desulfuromonas, Sulfurospirillum* and *Dehalococcoides*) (Maymó-Gatell *et al.*, 1997; He *et al.*, 2003b, 2005; Duhamel *et al.*, 2004; Smidt and de Vos, 2004; Sung *et al.*, 2006). However, only *Dehalococcoides* species are involved in the complete reductive dechlorination of PCE/TCE beyond DCEs to vinyl chloride (VC) and ethene (Smidt and de Vos, 2004). The functional reductive dehalogenase (RDase) genes in *Dehalococcoides* sp. code for proteins that catalyze these dechlorination steps, such as *pceA* (for PCE-to-TCE), *tceA* (for TCE-to-VC), *bvcA* and *vcrA* (for DCEs-to-ethene) genes (Magnuson *et al.*, 1998; Krajmalnik-Brown *et al.*, 2004; Müller *et al.*, 2004). In particular, *Dehalococcoides* sp. strains, BAV1 and FL2, are able to dechlorinate all DCE isomers to VC or ethene (He *et al.*, 2003b, 2005). Multiple *Dehalococcoides* strains have also been found in mixed cultures that work together to dechlorinate PCE completely to ethene (Hölscher *et al.*, 2004; Waller *et al.*, 2005; Holmes *et al.*, 2006). To date, the characterized PCE and TCE dechlorinators usually produce *cis*-1,2-dichloroethene (*cis*-DCE) predominantly and *trans*-1,2-dichloroethene (*trans*-DCE) negligibly (Smidt and de Vos, 2004). However, *trans*-DCE was found in at least 563 sites of the 1430 National Priority List Superfund sites identified by the US Environmental Protection Agency, whereas *cis*-DCE was detected only in 146 locations (ATSDR, 2007). Ratios of *trans*- /*cis*-DCE ranging from (1 to 7):1 have been documented in the TCE-contaminated subsurface of the Naval Air Force Station (Fort Worth, TX, USA) (http://pubs.usgs.gov/sir/2005/5176/) and in the TCE-contaminated wells of Key West, Florida

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(SWMU9, 2002). Significant amounts of *trans*-DCE were also detected at concentrations of up to 18 000 μg l^{-1} in groundwater samples taken from the upper tertiary aquifer in Bitterfeld (Germany) (Nijenhuis *et al.*, 2007). The accumulated *trans*-DCE in the chloroethene-contaminated sites tends to persist and disperse in the subsurface (He *et al.*, 2003a; Smidt and de Vos, 2004), hindering the complete removal of chloroethenes.

A few laboratory-scale studies reported that certain microorganisms in microcosms or mixed cultures could produce more *trans*- than *cis*-DCE during the reductive dechlorination of PCE/TCE (Löffler *et al.*, 1997; Griffin *et al.*, 2004; Miller *et al.*, 2005; Kittelmann and Friedrich, 2008). For instance, a number of uncultured microbes of the *Dehalococcoides* sp. and DF-1 in the *Chloroflexi* cluster were capable of producing *trans*- /*cis*-DCE in various ratios ((1.3–3.5):1) when fed with PCE/TCE (Futamata *et al.*, 2007). Although some *trans*-DCE may be produced through abiotic processes (Arnold and Roberts, 1998; ATSDR, 2007), a large fraction of accumulated *trans*-DCE at contaminated sites could be a result of microbial reductive dechlorination of PCE and TCE. Enrichment of such dechlorinating bacteria, in particular of the genus *Dehalococcoides,* has been proven to be laborious because of their long doubling time (>1 day). Therefore, limited information is available for dechlorinating microbes possessing specific RDase(s) involved in *trans*-DCE production and its further detoxification. So far, only *Dehalococcoides* sp. strain BAV1 could completely dechlorinate *trans*-DCE to the benign ethene (He *et al.*, 2003b). Owing to the persistent nature of *trans*-DCE and limited information on *trans*-DCE-dechlorinating bacteria, it would be of interest to look further into *trans*-DCE detoxification by multiple dechlorinators when *trans*-DCE was formed predominantly at the contaminated sites.

This study describes dechlorination of PCE/TCE to various amounts of *trans*-DCE by *Dehalococcoides*-containing microcosms, enrichment and a co-culture MB. The microbial populations associated with various *trans*- /*cis*-DCE generation ratios were investigated in detail. We have also identified the first *Dehalococcoides* sp. belonging to the Cornell subgroup responsible for *trans*- and *cis*-DCE production in culture MB. In contrast to previous reports linking the *Dehalococcoides* sp. to VC and ethene generation, in this study, we concluded that the specific *Dehalococcoides* sp., which produced *trans*-DCE predominantly, could not dechlorinate DCEs further to VC and ethene. However, complete dechlorination of PCE to ethene through predominant intermediate *trans*-DCE was achieved by co-inoculating the enrichment culture MB and a *trans*-DCE-dechlorinating culture 11a that contained multiple *Dehalococcoides* sp. The study of *trans*-DCE-producing consortia will provide more information on the diversity of dechlorinators involved in the generation of various dechlorination products (such as *trans*-DCE, VC or ethene) on exposure to PCE/TCE. These findings could then be extrapolated to contaminated sites and potentially aid in the determination of the type of bioremediation strategy, such as bioaugmentation, with the appropriate dechlorinators.

Materials and methods

Chemicals
The chlorinated ethenes, ethene, and other chemicals were purchased from Sigma-Aldrich (Saint Louis, MO, USA) with a minimum purity of 99.5%. Hydrogen was produced from a hydrogen generator (NM-H250, Schmidlin-DBS AG, Neuheim, Switzerland) and used as indicated.

Microcosm preparation
Sediments or slurry used for setting up microcosms were collected from San Francisco Bay Area (CA, USA), Sungei Buloh Wetland Reserve (Singapore), Wuhan industrial districts (Hubei, China) and Guiyu landfill sites receiving electrical waste (Guangdong, China). Triplicate microcosm studies were conducted in 60-ml serum bottles containing ~10 g of sediments or slurry, 25 ml autoclaved, bicarbonate-buffered mineral salts medium reduced with L-cysteine (0.2 mM), sodium sulfide (0.2 mM) and DL-dithiothreitol (0.5 mM) as previously described (Wolin *et al.*, 1963; Cole *et al.*, 1994; Löffler *et al.*, 1997; He *et al.*, 2002; He *et al.*, 2003a). The bottles were sealed with black butyl rubber septa (Geo-Microbial Technologies, Inc, Ochelata, OK, USA) and secured with aluminum crimp caps. All microcosms in triplicates were amended with 10 mM of lactate and 55 mM of PCE per bottle. Abiotic controls were carried out by autoclaving another set of microcosm bottles that were set up in the same way as the sample bottles. All bottles were inverted and incubated quiescently in the dark at room temperature.

Culture and growth conditions
After screening the above microcosms, two microcosms MB (San Francisco, CA, USA) and 11a (Hubei, China) were selected for further enrichment as they showed rapid dechlorination rates. After transferring 20 times in the presence of PCE or TCE, serial dilutions (10%, v/v) were used to enrich the cultures, which were carried out in 20-ml vials filled with 10 ml of mineral salts medium. In addition, ampicillin (50–300 mg l^{-1}) (Maymó-Gatell *et al.*, 1997; He *et al.*, 2003b) was added to the dilution series spiked with TCE (0.2 mM), acetate (10 mM) and hydrogen (500 000 ppmv). Initially, the cultures from the first three consecutive transfers received 200 mg l^{-1} of ampicillin. Subsequently, three consecutive dilution-to-extinction series and agar shakes were conducted. During these treatments, >50 colonies were picked up and re-inoculated

back to liquid medium to test their dechlorination activity on exposure to TCE. Dechlorination time-course studies were conducted in 160-ml serum bottles containing 100 ml of mineral salts medium amended with TCE or PCE (~50 μmoles, nominal concentration 0.5 mM), lactate (10 mM) or acetate (10 mM)/hydrogen (500 000 ppmv), a vitamin solution including 0.05 mg l^{-1} of vitamin B_{12} (He *et al.*, 2007), and 2% of inocula. All time-course studies were conducted in triplicates along with an extra abiotic control. All bottles were inverted and incubated quiescently in the dark at 30 °C.

Analytical methods
Chloroethenes and ethene were measured with a gas chromatograph (GC-6890, Agilent, Wilmington, DE, USA) equipped with a flame ionizing detector and a capillary column (GS-GasPro, 30-m length, 0.32-mm i.d., J&W Sci., Folsom, CA, USA). The oven temperature was initially held at 50 °C for 2 min, increased at 30 °C min^{-1} to 220 °C, and held for 1 min. The supernatants carrying volatile fatty acids (VFAs) were obtained by centrifugation of 1 ml of sample at 14 000 *g* for 10 min at 4 °C. VFAs were determined on a high-performance liquid chromatograph (Agilent 1100 HPLC system, Palo Alto, CA, USA) equipped with a UV detector (set at 210 nm). Separation of VFAs was conducted on an organic acid analysis column Aminex HPX-87 H (300 × 7.8 mm, Bio-Rad, Hercules, CA, USA) at 40 °C, and 5 mM H_2SO_4 was used as the eluent at a flow rate of 0.5 ml min^{-1}.

DNA extraction and PCR amplification
Cells for DNA extraction were collected periodically from 1 ml of culture samples by centrifugation (5 min at 20 000 *g*, 4 °C) in DNase/RNase-free microcentrifuge tubes. Cell pellets were stored at −20 °C until further processing. The genomic DNA was extracted with Qiagen DNeasy Tissue Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. PCR (Eppendorf, Hamburg, Germany) amplification was carried out by targeting the genomic DNA with universal Eubacterial primers 8F (Zhou *et al.*, 1995) and 1541R (Lane *et al.*, 1985) to obtain the 16S rRNA genes. Genomic DNA was also screened with the following species-specific primer pairs: *Dehalococcoides*, *Desulfuromonas* and *Dehalobacter* (Holliger *et al.*, 1998; Löffler *et al.*, 2000; Bunge *et al.*, 2003) as previously described. The primer sequences used in this study were included in the Supplementary material Table S1.

The presence of chloroethene RDase genes (*pceA*, *tceA*, *bvcA* and *vcrA*) in the cultures of this study was examined as previously described (Magnuson *et al.*, 1998; Krajmalnik-Brown *et al.*, 2004; Müller *et al.*, 2004). The PCR products were subsequently visualized on a Molecular Imager Gel Doc XR System (Bio-Rad) or on a Bioanalyzer using a DNA7500 Labchip Kit (Agilent Technologies Inc, Palo Alto, CA, USA).

The 16S rRNA genes copies of *Dehalococcoides* sp. in co-culture MB were determined by quantitative real-time PCR (qPCR) (ABI 7500 Fast Real-Time PCR system, Foster, CA, USA) as previously described (He *et al.*, 2003a, b; Sung *et al.*, 2006). Standard curves spanned a range of 10^2–10^7 gene copies per microliter of template DNA with a R^2 linear regression of 99.9%.

PCR–DGGE
PCR–denaturing gradient gel electrophoresis (PCR-DGGE) analyses were carried out for both universal bacterial 16S rRNA gene (with the primer pair 341F-GC and 534R) and *Dehalococcoides*-specific 16S rRNA gene fragments (1F-GC and 259R primer set) with a touch-down thermal program as previously described (Duhamel *et al.*, 2004). PCR-amplified fragments were electrophoresed on an 8% polyacrylamide gel with a 30–60% urea–formamide gradient for 16 h at 120 V and 60 °C.

Clone library
A clone library of 16S rRNA genes of co-culture MB was established by using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA) and all further clone-based experiments were carried out as previously described (Löffler *et al.*, 2000; He *et al.*, 2003a). The clones' purified plasmid DNA concentrations were measured by Nanodrop-1000 (NanoDrop Technologies Inc, Wilmington, DE, USA). The extracted plasmid DNA was subjected to restriction fragment length polymorphism analysis with the restriction endonucleases *Hha*I and *Msp*I (NEB, Ipswich, MA, USA). The 16S rRNA gene inserts representing groups of distinct enzyme restriction pattern were subsequently sequenced with an ABI 3100 Sequencer (Applied Biosystems, Foster City, CA, USA) by using primers M13F-20, M13R-24, 533F, 529R and 907F (http://www.genomics.msu.edu). Sequences were aligned with BioEdit assembly software (http://www.mbio.ncsu.edu/ BioEdit/bioedit.html) and were analyzed with BLASTN (http://www.ncbi.nlm.nih.gov/). The nearly complete *Dehalococcoides*-like 16S rRNA gene sequence (1489 bp) was deposited under the GenBank accession number EU073964. The other bacterium was characterized as being a *Sedimentibacter* species under GenBank accession number FJ593657.

Results

Dechlorination of PCE to predominant trans-*DCE*
From the 23 microcosms established with sediments collected from various locations, 3 (namely, MB, 11a and GY from CA, USA; Hubei, China; and Guangdong, China; respectively) generated more *trans*-DCE than *cis*-DCE in the reductive dechlorina-

Table 1 Summary of microcosms that produced *trans*-DCE and *cis*-DCE from dechlorination of PCE

Source	*Final product*	*trans-DCE/ cis-DCE ratio*
San Francisco Bay Area (CA, USA), MB	*trans*-DCE, *cis*-DCE	1.4 ± 0.1
Wuhan industrial districts (Hubei, China), 11a	Ethene (through *trans*-/*cis*-DCEs)	1.7 ± 0.2
Guiyu landfill sites (Guangdong, China), GY	*trans*-DCE, *cis*-DCE	2.8 ± 0.3
Sungei Buloh Wetland Reserve (Singapore), SB	*trans*-DCE, *cis*-DCE	0.4 ± 0.06

Abbreviations: *cis*-DCE, *cis*-1,2-dichloroethene; PCE, tetrachloroethene; *trans*-DCE, *trans*-1,2-dichloroethene.
Note: The presented ratios were from triplicate cultures with s.d.

tion of PCE, whereas SB (Singapore) generated relatively lower *trans*-/*cis*-DCE ratio (Table 1). No PCE dechlorination or lactate fermentation was observed in the abiotic control bottles (autoclaved microcosms). Microcosms, MB, SB and GY, generated *trans*-DCE and *cis*-DCE as the final dechlorination products, whereas 11a could dechlorinate PCE to ethene through intermediates *trans*- and *cis*-DCEs. Among these microcosms, MB exhibited the highest PCE dechlorination rate of 26.2 μmol l^{-1} day^{-1} with a *trans*- /*cis*-DCE generation ratio of (1.4 ± 0.1):1 and was selected for further transfers and investigation.

Enrichment culture MB was obtained by sequentially transferring the above PCE-to- *trans*/*cis*-DCE-dechlorinating microcosm. Methane was below detection limit after 10 transfers with PCE as substrate, though it was detected in the microcosms and the previous batches of sediment-free culture. The subsequent 10 transfers were fed with TCE and the time-course studies on the dechlorination of TCE were carried out thereafter. In defined mineral salts medium amended with lactate (10 mM) and TCE (0.2 mM), culture MB dechlorinated ~50 μmoles of TCE completely to *trans*-/*cis*-DCEs with a ratio of (1.43 ± 0.04):1 within 20 days, and a dechlorination rate of 30.6 μmol l^{-1} day^{-1} (Figure 1a). Dechlorination of TCE to *trans*- and *cis*-DCEs (in a ratio of 1.4:1) also occurred when culture MB grew in the same mineral salts medium amended with acetate (10 mM) only, or acetate (10 mM) and H_2 (170 000 ppmv). The sole acetate-grown culture (no H_2 supplied) showed the slowest dechlorination rate. Thereafter, the acetate-grown sub-culture was used as inocula for subsequent transfer to fresh acetate and H_2 medium so as to facilitate the enrichment and isolation of the dechlorinators.

When culture MB was treated with ampicillin (50 mg l^{-1}) in the mineral salts medium amended with acetate and H_2, dechlorination of TCE occurred at a slower rate as compared with dechlorination without ampicillin. The treatment of acetate/H_2 cultures with 0–300 mg l^{-1} of ampicillin resulted in the variation of *trans*-DCE to *cis*-DCE ratio, ranging from (1.4 to 3.0):1. The highest ratio was achieved in the culture receiving 200 mg l^{-1} of ampicillin dosage. After growing in ampicillin-amended medium for

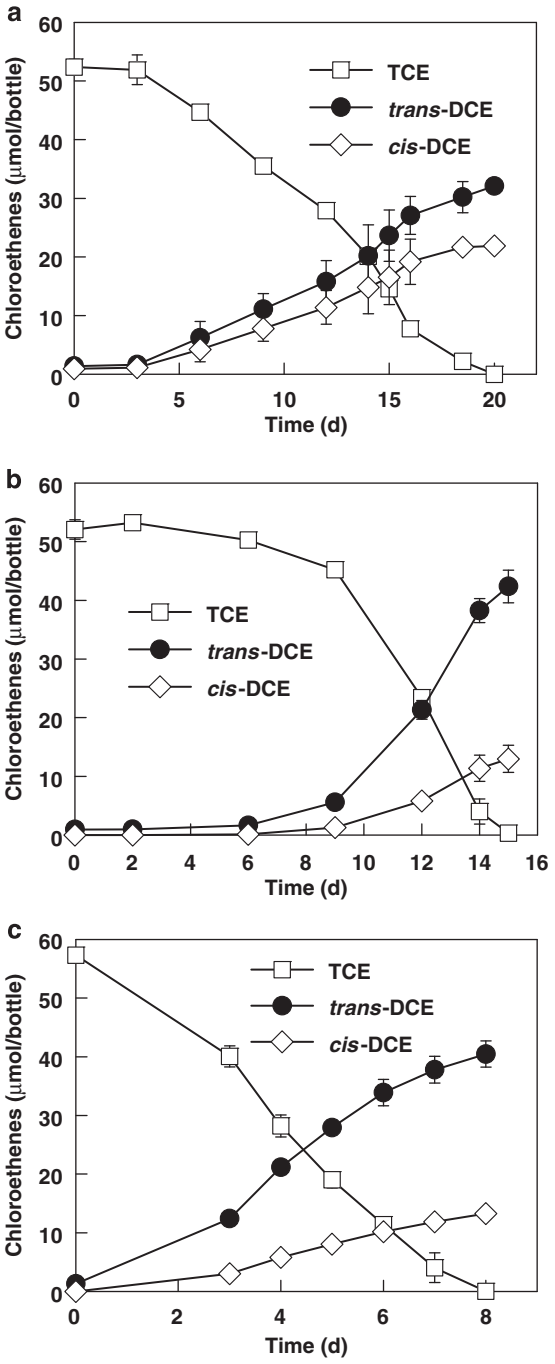

Figure 1 Reductive dechlorination of trichloroethene (TCE) to *trans*-1,2-dichloroethene (*trans*-DCE) and *cis*-1,2-dichloroethene (*cis*-DCE) by culture MB at different enrichment phases. (**a**) Enrichment culture MB after 20 transfers in lactate-amended medium. (**b**) Further enrichment culture MB grown with acetate (10 mM) and H_2 (260 000 ppmv) after ampicillin treatment. (**c**) Highly enriched culture MB after three more transfers in acetate-/H_2-amended medium. No vinyl chloride, ethene, ethane or methane was detected on extended incubation. Datum points were averaged from triplicate cultures. Error bars indicate s.d. and are not shown when they are smaller than the symbol.

two more transfers, the active cultures were then continuously transferred to medium amended with acetate and H_2 only. Consequently, culture MB was capable of dechlorinating ~50 μmoles of TCE to *trans*- and *cis*-DCEs with an increased ratio of 3.7:1 (Figure 1b) within 15 days. However, when culture MB was transferred back to the mineral salts medium amended with lactate, an extended lag phase (~13 days) was observed and the dechlorination of TCE (~50 μmoles) took another 17 days in all the triplicate bottles (data not shown). Acetate and propionate were detected as the fermentation products measured with high performance liquid chromatograph, indicating that certain fermentative microbes had yet to be diluted out of the culture.

After three more transfers in the absence of antibiotics, the ampicillin-treated culture MB with acetate and H_2 achieved its highest TCE dechlorination rate (68.8 μmoles l^{-1} day^{-1}) but the *trans*-/*cis*-DCE generation ratio remained unchanged (Figure 1c). Culture MB was continuously transferred to fresh medium (every 3–4 weeks) over a period of 2 years and showed consistent PCE/TCE dechlorination rates and *trans*-/*cis*-DCE generation ratio. At this stable dechlorination stage, culture MB was observed under light microscopy, which showed the prevalence of small disc-shaped bacteria and a lower abundance of rod-shaped bacteria. The size of the disc-shaped microbes coincides with that of the reported *Dehalococcoides* sp. (~1.0 μm in diameter). Attempts had been made to isolate each of them by both serial dilutions and agar shakes amended with acetate and H_2. However, only the rod-shaped bacteria were able to grow in pure culture successfully. Its 16S rRNA gene sequence was shown to be closely (99% identity over 1504 bp, 5 bp difference) related to *Sedimentibacter* sp. C7, which was reported to grow in co-culture with a *Dehalobacter* species (van Doesburg *et al*., 2005). This isolated *Sedimentibacter* sp. did not show dechlorination activity on PCE or TCE. Therefore, culture MB should mainly consist of the PCE-dechlorinating *Dehalococcoides* and the *Sedimentibacter* sp.

Identification of the trans-*DCE-producing microbes*

To identify the dechlorinating microbes in the sediment-free cultures, MB, 11a, GY and SB, genus-specific primers targeting the 16S rRNA genes of *Dehalobacter, Desulfuromonas* and *Dehalococcoides* were tested on genomic DNA of the above cultures by PCR. When targeted with *Dehalococcoides*-specific primers, the PCR amplicons yielded the expected DNA size of 620 bp for all the cultures (data not shown). When using *Desulfuromonas*-specific primers to amplify the genomic DNA of the above *trans*-DCE-producing cultures, only culture MB exhibited a very weak band of 815 bp. However, the *Desulfuromonas*-like species was no longer detected in MB when the ratio of *trans*- to *cis*-DCE reached ~3.7:1. Amplification of the genomic DNA using *Dehalobacter*-specific primers yielded no amplicons for all the cultures. Therefore, the PCR analysis based on 16S rRNA genes suggests that the rod-shaped *Desulfuromonas* or *Dehalobacter* species may not contribute to *trans*-DCE formation, and further confirm the existence of *Dehalococcoides*-like species in the above cultures.

Dehalococcoides *species diversity versus the ratio of* trans- *to* cis-*DCE*

To understand the elevated ratio (1.4:1–3.7:1) of *trans*-/*cis*-DCE in culture MB, PCR-DGGE was used to track the community structures with universal bacterial primer pair and the diversity of *Dehalococcoides* sp. with genus-specific primer pair by targeting the genomic DNA of culture MB at different enrichment stages. Figure 2a shows the microbial community transitions from mixed culture (before ampicillin treatment), highly enriched culture (after one-time ampicillin treatment), to apparent co-culture (after ampicillin treatment), reflected by detecting multiple DGGE bands to two major bands. The band on the top was getting thicker, which turned out to be *Dehalococcoides* sp. This was verified by the positive control, plasmid DNA containing the 16S rRNA gene fragment of *Dehalococcoides ethenogenes* strain 195. The second major band in Figure 2a corresponded to the minor group in the co-culture, *Sedimentibacter*, which was confirmed by the positive control of this isolate. It is to be noted that the *Desulfuromonas*-like band disappeared in the post-amplicillin-treated MB cultures (Figure 2a). Only one band representing the Cornell subgroup of *Dehalococcoides* sp. (Figure 2b) was detected in the *trans*- and *cis*-DCE-producing culture MB. Therefore, the detected *Desulfuromonas*-like species before amplicillin treatment (Figure 1a) could have contributed to *cis*-DCE production in the mixed culture MB. In all, the DGGE profiles confirmed that culture MB consisted of the Cornell subgroup of *Dehalococcoides* and *Sedimentibacter* only, whereas the former was responsible for *trans*-DCE production from PCE/TCE.

Sediment-free cultures obtained from microcosms shown in Table 1 also generated *trans*- and *cis*-DCEs in various ratios. The presence of multiple *Dehalococcoides* sp. bands (for example, GY and 11a) explained the various ratios of *trans*-/*cis*-DCE, as shown in this study (Figure 2c). Figure 2c also shows the stronger the MB-like bands, the higher *trans*-/*cis*-DCE generation ratios in the sediment-free cultures; conversely, the stronger the FL2-like bands, the lower the *trans*-/*cis*-DCE ratios in these cultures.

Clone library and sequence analysis of culture MB

To further determine the phylogeny of the PCE-to-*trans*/*cis*-DCEs dechlorinator(s), a clone library based on 16S rRNA genes was established with

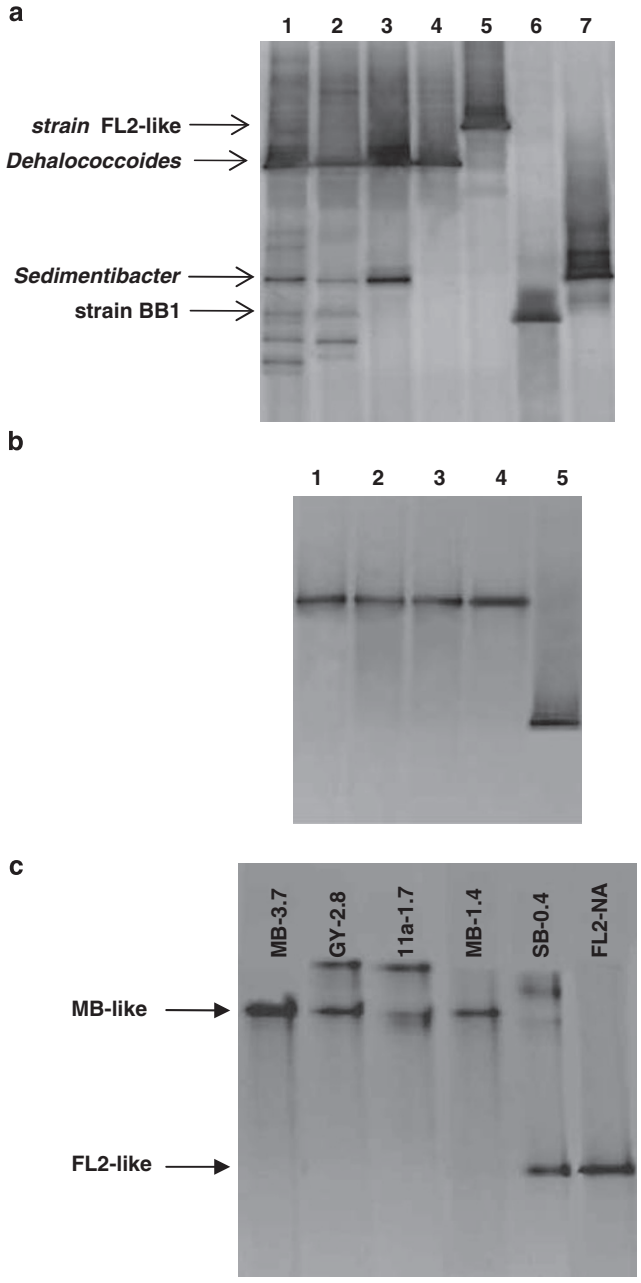

Figure 2 PCR–denaturing gradient gel electrophoresis (PCR-DGGE) profiles of *trans*-1,2-dichloroethene (*trans*-DCE)-producing cultures. (**a**) DGGE profile of PCR amplified with a universal bacterial primer pair targeting genomic DNA of culture MB. (**b**) DGGE profile of PCR amplified with a *Dehalococcoides*-specific primer pair targeting genomic DNA of culture MB. The template DNA for lane 1, 2 and 3 was extracted from cultures as shown in the time-course studies of Figures 1a–c, respectively. Lane 1, culture MB before ampicillin treatment; lane 2, culture MB after one-time ampicillin treatment; lane 3: culture MB after ampicillin treatment; lane 4, positive control of *D. ethenogenes* 195 clone DNA; lane 5, a trichloroethene (TCE)-to-*cis*-1,2-dichloroethene (*cis*-DCE)-to-vinyl chloride culture, which shared 100% 16S rRNA gene as *Dehalococcoides* species strain FL2; lane 6, *Desulfuromonas* species strain BB1; lane 7, an isolate of *Sedimentibacter*. (**c**). PCR-DGGE profile of *Dehalococcoides* sp. present in different TCE-dechlorinating cultures. The number on the top of the lane indicates the ratio of *trans*-DCE to *cis*-DCE produced from various microcosms as described in Table 1. *Dehalococcoides* sp. strain FL2-like culture serves as a control. NA, no *trans*-DCE was accumulated during the dechlorination of TCE.

the genomic DNA extracted from the apparent co-culture MB fed with acetate and H_2. The restriction fragment length polymorphism analysis of 57 clones revealed two different digestion patterns (56:1). Twenty-eight of the 56 clones, and the last single clone were sequenced for their 16S rRNA gene inserts in the plasmids. After aligning the sequences with the BioEdit assembly software, the contigs were analyzed using BLASTN and compared with known existing 16S rRNA gene databases. Results showed that the 56 clones possessed identical 16S rRNA gene sequence, which shares 100% identity of the 16S rRNA gene sequence of *D. ethenogenes* strain 195 (accession number CP000027) from its complete genome. The 16S rRNA gene sequence of the uncultured *Dehalococcoides* sp. strain MB (the Cornell subgroup of *Dehalococcoides* sp.) was deposited in GenBank under accession number of EU073964 (1489 bp). In addition to the above *Dehalococcoides* sp. found in the culture MB, the sequence of the other clone showed that it belonged to the genus of *Sedimentibacter* as isolated in pure culture.

The presence of the known RDase genes was also examined in the *trans*- and *cis*-DCE-producing cultures. The following genes, *pceA*, *tce*A, *bvcA* or *vcrA*, were not detected in culture MB when fed with PCE or TCE. The results suggested that a novel RDase gene, instead of *pceA* or *tceA* gene, might be responsible for the production of *trans*-DCE by the *Dehalococcoides* in culture MB. Therefore, on the basis of the above analysis (for example, constant *trans*- /*cis*-DCE ratio, light microscopy, DGGE, clone library, RDase genes), we were able to ascertain that the culture was further enriched to a co-culture MB consisting only of *Dehalococcoides* sp. of the Cornell subgroup and *Sedimentibacter* sp.

Growth of Dehalococcoides*-like species in co-culture MB*

Quantitative real-time PCR was carried out to further confirm that *Dehalococcoides*-like species was responsible for the generation of *trans*-DCE during the dechlorination of TCE. The *Dehalococcoides* cells were measured by targeting the genomic DNA extracted from co-culture MB. qPCR results showed the increase of *Dehalococcoides* 16S rRNA gene copies with the dechlorination of TCE (Figure 3). The total *Dehalococcoides* cell number reached 2.54×10^8 cells per ml from the initial 5.91×10^6 cells per ml ($\sim$43 times increase) in the co-culture, verifying that the increase of the *Dehalococcoides* 16S rRNA gene copies was directly correlated with the dechlorination of TCE to the predominant *trans*-DCE.

Complete dechlorination of TCE to ethene through trans- *and* cis*-DCEs by culture MB and 11a*

Similar to culture MB, the sequential transfers of another microcosm (Hubei, China) generated

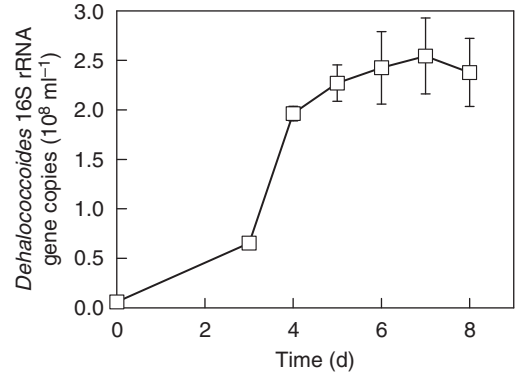

Figure 3 The growth of the *Dehalococcoides* species with the dechlorination of trichloroethene to predominant *trans*-1,2-dichloroethene by co-culture MB.

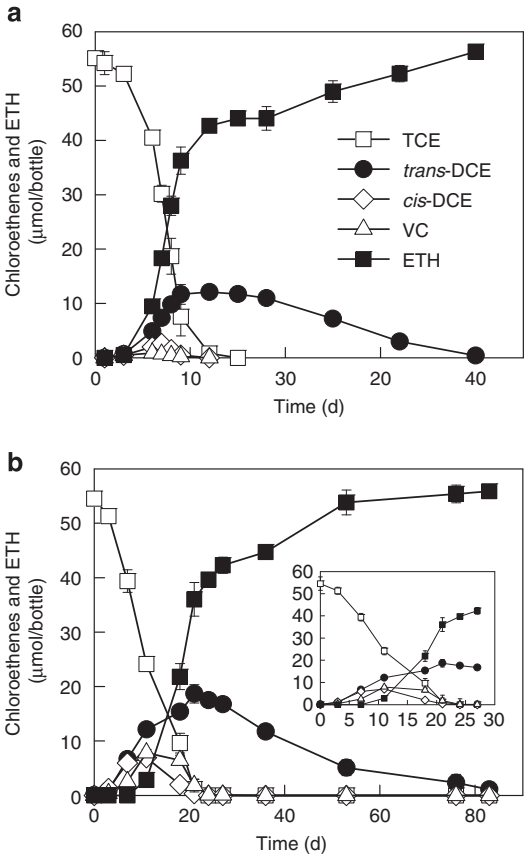

Figure 4 Reductive dechlorination of trichloroethene (TCE) to ethene through *trans*-1,2-dichloroethene (*trans*-DCE) predominantly. (**a**) Culture 11a alone amended with lactate. (**b**) Culture MB and 11a amended with acetate (10 mM) and H_2 (400 000 ppmv). (Datum points were averaged from triplicate cultures. Error bars indicate s.d. and are not shown when they are smaller than the symbol). Figure 4b shares the same symbols as Figure 4a.

enrichment culture 11a, which was capable of completely dechlorinating TCE to ethene through intermediates *trans*- and *cis*-DCEs (in a ratio of 1.7:1) in lactate-amended mineral salts medium (Figure 4a). After 40 days, the generated DCEs were completely dechlorinated to ethene. DGGE profile showed that culture 11a contained multiple *Dehalococcoides* sp. (Figure 2c), one of which was MB-like to produce *trans*-DCE, whereas another may function to further dechlorinate *trans*-DCE. With the *trans*-DCE-dechlorinating mixed culture 11a in hand, it was inoculated together with culture MB to the bottles spiked with TCE to examine the possibility of complete reductive dechlorination of the persistent *trans*-DCE generated biologically by culture MB. As shown in Figure 4b, complete dechlorination to ethene through *trans*-DCE occurred within 80 days. The maximum *trans*- to *cis*-DCE ratio reached ~1.75:1 on day 11 when *cis*-DCE concentration started to decrease. During the reductive dechlorination process, *trans*-DCE was found to be the main intermediate, accumulating up to 18.6 μmoles on day 21, whereas small amounts of *cis*-DCE and VC (about 6.9 and 7.8 μmoles, respectively) appeared and the amount of *1,1*-DCE produced was negligible. After day 21, *trans*-DCE started to be dechlorinated to ethene without the accumulation of VC.

Discussion

In this study, a highly enriched PCE-to-*trans*/*cis*-DCE-dechlorinating co-culture MB was characterized and the growth of the newly identified *trans*-DCE-producing *Dehalococcoides* sp. was verified by qPCR. A number of identified *trans*-DCE-producing *Dehalococcoides* sp., including the Cornell (in this study) and Pinellas (Griffin *et al.*, 2004) subgroups, showed a common inability to dechlorinate PCE/TCE beyond DCEs to VC and ethene. The dechlorination pathway of MB is different from the previously discovered *Dehalococcoides* sp. linking to VC or ethene production from polychloroethenes, including those that produced predominant 1,1-DCE isomers during the reductive dechlorination of TCE (Zhang *et al.*, 2006). Complete detoxification of PCE/TCE through intermediates *trans*- and *cis*-DCEs also occurred when the required dechlorinating microbes were present in the community, as shown in the mixed culture of 11a and MB. The results from this study indicate that accumulation of *trans*-DCE through microbial reductive dechlorination in contaminated sites could be significant when the *trans*-DCE dechlorinator(s) are absent or are operating at a lower rate compared with that of *cis*-DCE and VC dechlorinators. The chloroethene distribution in contaminated sites is significantly affected by different types of indigenous dechlorinators (Smidt and de Vos, 2004). The *trans*-DCE-generating microbe discovered in this study would enhance the current understanding of diverse dechlorinating capabilities of the *Dehalococcoides* genus.

During the enrichment process from sediment-free culture to co-culture MB, the ampicillin-treatment step significantly increased the culture's TCE dechlo-

rination rates (from 26.2 to 68.8 μmoles l^{-1} day^{-1}) and the ratio of *trans*-DCE to *cis*-DCE (from (1.4 ± 0.1):1 to (3.7 ± 0.4):1). Previous studies showed that sediment-free cultures generated *trans*-DCE to *cis*-DCE in ratios of (3 ± 0.5):1 (Griffin *et al.*, 2004) and (1.3 ± 0.2):1 (Miller *et al.*, 2005), accompanying with an average TCE dechlorination rate of 4.8 μmoles l^{-1} day^{-1} (Griffin *et al.*, 2004) and 20 μmoles l^{-1} day^{-1} (Miller *et al.*, 2005), respectively. The increased dechlorination rates and *trans*- to *cis*-DCE ratios for culture MB could be explained by that (i) the competitors for electron donor H_2 (for example, methanogens) were diluted out; (ii) culture MB contained higher number of *trans*-DCE-producing *Dehalococcoides* cells, but less or negligible amount of the *cis*-DCE-producing *Desulfuromonas*-like cells (revealed by DGGE profiles), compared with the previous cultures. This is also supported by the fact that the majority of the clones (56 out of 57) belong to *Dehalococcoides* and the *Desulfuromonas* band disappeared in the co-culture MB. However, culture MB was not a pure culture yet as lactate fermenters (for example, *Sedimentibacter*) were still present. This is reflected by their recovered dechlorination activity after an extended incubation time when fed from acetate/H_2- back to lactate-amended medium.

By sequencing of the representative clones (established with PCR 16S rRNA gene inserts), *Dehalococcoides* sp. was identified to be responsible for PCE/TCE dechlorination to *trans*- and *cis*-DCEs. On the basis of the 16S rRNA gene sequence similarity, *Dehalococcoides* sp. MB belongs to the Cornell subgroup of the *Dehalococcoides* cluster (Hendrickson *et al.*, 2002), which is different from the *trans*- and *cis*-DCE-producing *Dehalococcoides* populations in the Pinellas group (Griffin *et al.*, 2004) (refer to Supplementary Table S2). Supplementary Table S2 also shows that bacterium DF-1 and TFCC groups (T-RF 513 bp and T-RF 143 bp), which are distantly related to *Dehalococcoides* sp., contributed to the production of *trans*-DCE. We could not exclude the possibility of other bacteria having a role in *trans*-DCE formation in the environment. However, this study provides conclusive proof that the Cornell subgroup of *Dehalococcoides* sp. in co-culture MB grew with the dechlorination of TCE to predominant *trans*-DCE by both qualitative (for example, DGGE and clone library) and quantitative (real-time PCR) molecular tools. On the other hand, owing to the high similarity of 16S rRNA gene sequences for the genus *Dehalococcoides*, it is difficult to rule out the presence of other *Dehalococcoides* sp. Nevertheless, the Cornell subgroup of *Dehalococcoides* has never been shown to have this dechlorination ability of producing *trans*-DCE predominantly. The stable *trans*-/*cis*-DCE ratio, two morphologies by light microscopy and the culture-independent approaches (DGGE, clone sequencing, RDase gene analysis) strongly suggest that co-culture MB contained only one *Dehalococcoides* sp. The interactions between *Dehalococcoides* and *Sedimentibacter* are not clear but may be nutritional in nature. Isolation of this particular strain of *Dehalococcoides* will lead to an improved understanding of the nutritional requirements and physiological characterization of this novel *Dehalococcoides* sp. Therefore, this study and previous reports (Griffin *et al.*, 2004; Miller *et al.*, 2005; Kittelmann and Friedrich, 2008) suggest that these diverse *trans*-DCE-producing dechlorinators belong to the green non-sulfur bacteria, including *Dehalococcoides*, the Tidal Flat Chloroflexi cluster and bacterium DF-1.

Despite the high conservation of the 16S rRNA gene among the *Dehalococcoides* sp., RDase genes (*pceA*, *tceA, bvcA* and *vcrA*) have been identified to differentiate reductive dechlorination of chloroethenes (Magnuson *et al.*, 1998; Krajmalnik-Brown *et al.*, 2004; Müller *et al.*, 2004). In the enrichment process of culture MB, no *pceA* or *tceA* gene was detected in its genomic DNA. This observation suggests that the *tceA* gene in *D. ethenogenes* 195 is different from the functional *trans*-DCE-producing gene(s) in the *Dehalococcoides* sp. of culture MB. Similarly, bacterium DF-1 was reported to dechlorinate PCE/TCE to significant amounts of *cis*- and *trans*-DCEs, whereas no *tceA* gene was reported in its genome (Miller *et al.*, 2005). Therefore, the *trans*-DCE-producing gene could be quite different from the currently identified RDase genes, which may also be potentially acquired through horizontal gene transfer as suggested for the known RDases in *Dehalococcoides* sp. (Regeard *et al.*, 2005; Seshadri *et al.*, 2005). PCR-DGGE identified a common band, whose presence may indicate *trans*-DCE production from PCE or TCE. However, the detection of this band in DGGE would not guarantee the generation of *trans*-DCE in the *Dehalococcoides* sp., for example, *D. ethenogenes* strain 195.

Findings in this and previous studies suggest that diverse *trans*-DCE-producing *Dehalococcoides* sp. are present at chloroethene-contaminated sites and these *Dehalococcoides* sp. are capable of producing high levels of *trans*-DCE (Löffler *et al.*, 1997; Griffin *et al.*, 2004; Miller *et al.*, 2005; Futamata *et al.*, 2007; Kittelmann and Friedrich, 2008). For the first time, we reported that the *trans*-DCE-producing *Dehalococcoides* sp. was unable to generate VC and ethene during the dechlorination process through characterization of the co-culture MB (~98% of the bacteria belonging to the Cornell subgroup of *Dehalococcoides* sp. and ~2% affiliated with *Sedimentibacter*), whereas previous *Dehalococcoides* cultures were linked to VC and ethene generation. Hence, the large amounts of *trans*-DCE detected in the TCE-contaminated sites might be a result of the presence of *trans*-DCE-producing microbes and the lack of *trans*-DCE dechlorinators. Fortunately, culture 11a in this study proves to be a promising candidate in the complete dechlorination of PCE/ TCE to harmless ethene through the cooperation of multiple *Dehalococcoides* sp. Thus great care must be taken

when bioremediation techniques are being applied to such *trans*-DCE-formation sites, as the *trans*-DCE-producing culture (for example, MB) is also capable of using the same electron donor as the other *Dehalococcoides* strains, such as BAV1. The identification and administration of the microbe(s) responsible for rapid and complete dechlorination at any particular chloroethene-contaminated site may provide a better bioremediation strategy.

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